CHEMOTROPISM IN RHIZOPUS NIGRICANS. II. THE ACTION OF PLANT JUICES

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Parasitic fungi invade the tissues of their host plants by sending their growing hyphal tips through the stomata of the leaves or by actually piercing the epidermis. To pass through a stoma, the hypha must first grow to a particular point on the leaf surface. To penetrate the epidermis, it must break through a barrier. Investigators have long believed that such non-random patterns of growth must involve some directive influence by the host plant. Such influence seems even more necessary in view of the high specificity shown by some parasites—they will invade the tissues of only a particular kind of host. The question of how a host plant could direct the growth of its parasite led to some early studies of chemical tropism in fungi.

The first work dealing directly with this problem was that of Miyoshi (1894), in which he sowed spores of several molds on the under surface of Tradescantia leaves which had been injected with various chemical substances. These substances presumably diffused out through the stomata and onto the surface. He reported that when the leaves contained proper concentrations of certain nutrients (particularly sugars and meat extracts), the hyphae grew directly to and through the stomata. On leaves injected only with water, he found the hyphae to grow in random directions, while if the leaves contained harmful substances (acids, alkali, alcohol and certain salts), growth was oriented away from the stomata. He observed the same tropic responses when he sowed the spores on mica plates with pin holes in them, the substance under test being in a gelatin layer in contact with the opposite face of the plate. Thus Miyoshi concluded that fungi exhibit an extensive (and extremely useful) chemotropic sensitivity, guiding them to supplies of nourishment and away from damaging materials.

The case for chemotropic control of parasitic molds was expanded by Massee (1905). He confirmed Miyoshi's finding that various fungi grow tropically toward sugars. But he found obligate parasites to be attracted only by decoctions from their own hosts. He believed facultative parasites to be attracted by sugar, but repelled by certain other substances, which prevented them from invading certain types of hosts (thus he said Botrytis failed to grow into green apples because of a negative tropic sensitivity to malic acid). Massee concluded that the specificity of parasitism was based completely on chemotropism. His findings have not been confirmed in any other work, to our knowledge. Certain substances for which he reported strong chemotropic action have been tested on Rhizopus in the course of the current study, but always with negative results.

The findings of Miyoshi were checked by Clark (1902), using the injected Tradescantia leaves, but with quite different results. He wished particularly to test the suggestion of Swingle (1896) that sprays of copper compounds protect host plants through a negative chemotropic action on parasitic fungi. He concurred with Miyoshi in the finding that the hyphae grew into Tradescantia stomata toward sugars, but he found them to grow in just as readily if the leaf had been injected with plain water or even with toxic copper salts. Likewise, on mica plates, the hyphae grew into the hole regardless of what substance was on the other side. The only condition which prevented growth toward the hole was the presence of germinating spores in the layer on the other side. He concluded that the germ tubes growing toward the holes when no spores were present on the other side must have tropic sensitivity to some product of the mold metabolism and must be growing *away* from higher concentrations of this material. This growth behavior has been called the "staling reaction," as it is growth away from regions of stale medium. Clark's conclusion that molds have a negative tropic sensitivity to some product of their own metabolism has been adequately confirmed by later studies (Fulton, 1906; Graves, 1916; Stadler, 1952).

There has been some disagreement as to whether sugars and other nutrients exercise a positive chemotropism on molds. Miyoshi (1894) reported pronounced turning of the hyphal tips toward concentrations of various nutrients, but Clark (1902) maintained that the oriented growth was caused only by the negative tropic effect of the staling factor, and that hyphae could grow just as readily into water or harmful substances as into nutrients. This was the view also of Fulton (1906), who looked for tropic action by a large number of substances on eight different molds; he detected no positive tropism and concluded that orientation was based solely on the staling reaction. Graves (1916) recognized that the staling reaction caused the most marked orientation, but he reported that hyphae turned more pronouncedly toward a sugar-containing medium than toward plain water; thus, he concluded that sugar does exercise an attraction, but that it is normally masked by the stronger tropic action of the staling substance.

Some of the experiments on sugars were repeated during the present study in an attempt to determine whether or not there was any tropic action by these substances. The interpretation of such studies is complicated by the fact that staling-substance production (and thus orientation caused by the staling reaction) varies with sugar concentration. When the experiments were designed in a way to minimize variation in the staling reaction, the results gave some indication of a very slight attraction by glucose and by sucrose, but they were not conclusive.

Graves reported that turnip juice medium had a much more powerful positive tropic effect than sugars, though again, not as strong as the staling reaction. The turnip juice finding was reminiscent of the report of Massee (1905) and other early suggestions that tropic attraction by plant juices might be responsible for host penetration by parasitic fungi.

In the present study the tropic action of turnip juice and other plant materials on germinating spores of *Rhizopus nigricans* has been extensively studied. These materials cause striking orientation, but further investigation of this phenomenon has led us to the conclusion that this orientation is not the result of a direct tropic action on the mold by the plant juices.

METHODS

The method used in most of the experiments is the same as described previously (Stadler, 1952); plates of plastic coverslip material with circular holes drilled in

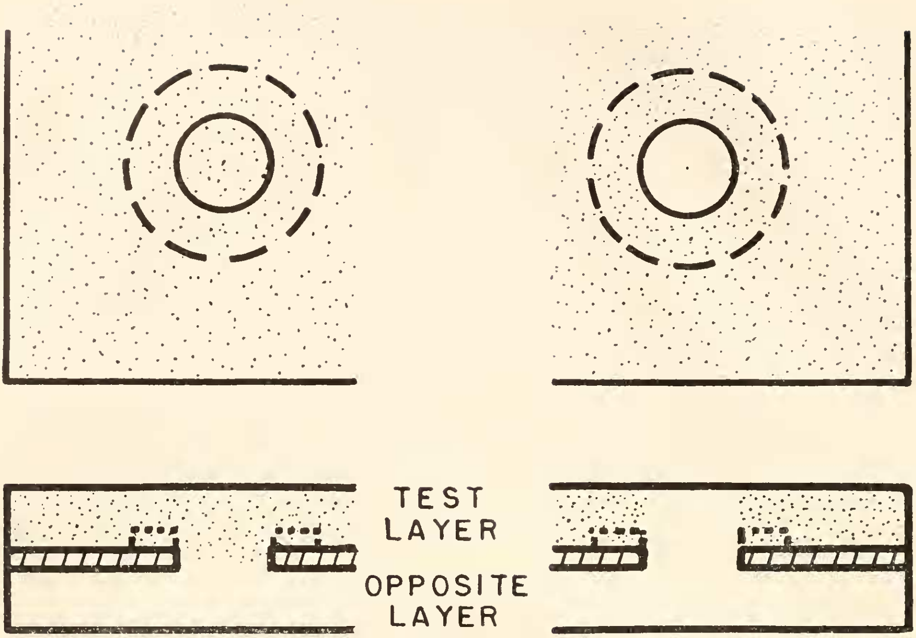

Figure 1. Enlarged representations of the corner of a plate showing one hole in face view and in profile. On the left is the plate as prepared in the ordinary tests. The dots represent the spores of the test layer, and the broken line shows the limits of the test region. The picture on the right shows a preparation made by the "thumb tack" method, whereby no spores are present within or above the hole and the test region becomes the edge of the population.

them are used. A layer of agar medium is placed on each side of the plate; the agar layers are in contact with each other only within the holes. Any material contained in one layer but not in the other will tend to diffuse through the hole, setting up a concentration gradient in the region in and near the hole. One of the layers, in every case, contains a suspension of spores of Rhizopus. This is the "test layer." The direction of growth is studied of the spores in the test layer in the region immediately around the hole. The other layer is called the "opposite layer" and may or may not contain spores, depending on the experiment being done. The preparations are incubated for eight and one half hours at 28° C. and then fixed and stained. Camera lucida drawings are made of the spores in each of the "test regions" (washer-shaped region circumscribing the hole, Fig. 1), and the directions of growth are measured. The angle measured is the direction of growth of the germinating spore with respect to the direction of the chemical gradient. Any gradient set up between the two layers must be oriented directly out from the center of the hole. When a germ tube grows directly toward the center of the hole, the angle recorded is zero; when it grows directly away from the hole, the angle is 180 degrees.

If the germ tubes of spores in a particular experiment are growing in random directions, then the average of the angles for all the spores in a test region should be about 90 degrees. This is the result when we do an experiment with spores in nutrient medium in the test layer and the same concentration of spores in the same medium in the opposite layer. (The synthetic medium used in these experiments contained 1% glucose, 10^{-2} M aspargine, 10^{-2} M KH_2PO_4, and 2×10^{-3} M $MgSO_4$.) When a plate is prepared with spores in the nutrient medium in the test layer, and the opposite layer is made up with the same medium (or even water agar) but with no spores, then the spores in the test regions grow toward the holes. If the test layer contains 120 spores per mm.^{3}, the average angle will be 40–45 degrees (Fig. 2-A). This is the staling reaction—growth away from populated regions, away

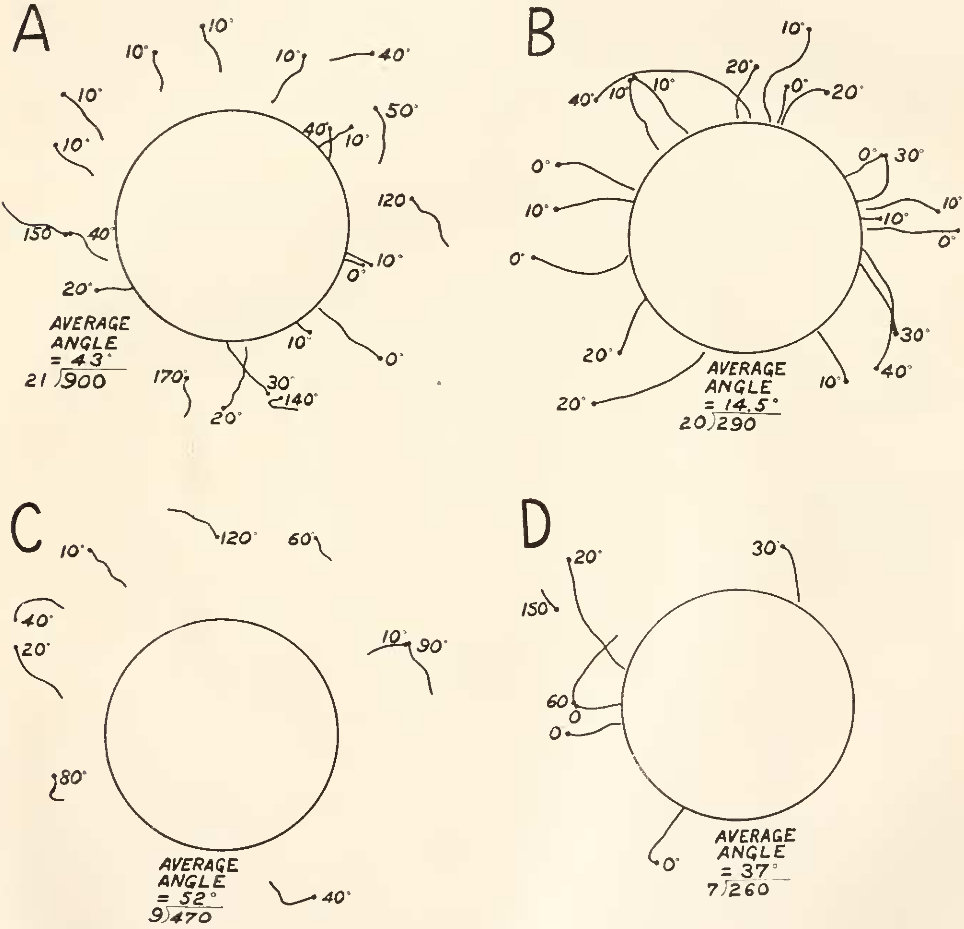

FIGURE 2. Camera lucida drawings of test regions from experimental plates showing the improvement of orientation elicited by plant juices in the opposite layer. *A:* 120 spores per mm.^{3} in the synthetic medium in the test layer, non-spore synthetic medium in the opposite layer; *B:* 120 spores per mm.^{3} in synthetic medium in the test layer, non-spore elm leaf decoction in the opposite layer; *C:* 40 spores per mm.^{3} in synthetic medium in the test layer, non-spore synthetic medium in the opposite layer; *D:* 40 spores per mm.^{3} in the synthetic medium in the test layer, non-spore elm leaf decoction in the opposite layer.

from concentrations of the unknown metabolic product (or products) called the staling substance.

THE "ATTRACTION" OF PLANT JUICES

If turnip juice is mixed into the non-spore opposite layer of a plate with spores in synthetic medium in the test layer, all the spores in the test region will germinate and grow toward the hole. The orientation is much more marked than when synthetic medium is used in the non-spore opposite layer (Fig. 2-A, B); in that case the average angle was 40–45 degrees; with turnip juice in the opposite layer it is 10–20 degrees.

This same "attraction" is elicited by several other natural mixtures from plant sources. Yeast extract in the opposite layer causes striking orientation toward the hole. Tomato juice does the same, as does a decoction of elm leaves. A strain of Penicillium has been isolated which imparts this property to the synthetic medium when grown in that medium for one week. (This strain has been identified by Dr. Kenneth B. Raper as *Penicillium spinulosum* Thom.) The young growing mycelium of Rhizopus itself if heated to 60° C. for one hour, releases into the medium around it a material with this same effect. These "attractants" from various sources are all alike in that they are water-soluble and heat stable, and all evoke the same marked orientation of Rhizopus on the plates used in this study. Furthermore, they are alike in that there is evidence in each case that the material does not exercise a true chemotropic attraction on the Rhizopus spores.

TABLE I

Comparison of "attraction" effect in high and low spore concentrations

"Attractant"	Spore concentration in test layer (per mm.^{3})	Average Angle		Average improvement of orientation (in degrees) by "attractant"
		With non-spore synthetic medium in opposite layer	With "attractant" added to nonspore opposite layer	
Elm leaf decoction	120	45 degrees	17 degrees	28
	40	54 degrees	38 degrees	16
Cooked Rhizopus (medium in which Rhizopus has been grown and heated to 60% C. for one hour before being filtered off)	120	47 degrees	27 degrees	20
	40	55 degrees	46 degrees	9
Yeast extract	120	42 degrees	22 degrees	20
	40	57 degrees	42 degrees	15

Note: Each average angle given in this table is the mean value of four test regions.

The suspicion arose that these materials might not exert a simple attraction on Rhizopus when it was noted that a preparation which markedly improved the orientation on plates with high concentrations of spores in the test layer had much less effect on orientation on plates with low spore concentrations (Table I, Fig. 2).

This suggested that the action of these materials might be somehow related to the staling reaction, since the intensity of this reaction varies with spore concentration. If the material were a true attractant, it should act on spores in its presence regardless of the intensity of the staling reaction.

Orientation, in these experiments, is normally limited by a shortcoming of the method. The strongest orientation elicited by the staling reaction should be shown by the spores on the *edge* of the populated region, as this is where the concentration of staling substance is diminishing most markedly. The spores in the test regions of these plates are not on the edge of the population, but near the edge. There are always some spores *within* the hole—this is the edge of the population mass; the test region is back from the edge (Fig. 1). When an experiment is done with a sparse population of spores in the test layer, there are very few spores within the hole, and these have a minimal effect on the amount of orientation. But in heavy populations the spores within the hole become important, as there are enough of them to produce considerable staling substance and seriously limit the orientation in the test region. The extent of orientation depends upon the steepness of the concentration gradient of staling substance in the test region. If it is being produced in large amounts not only on the side away from the hole, but also on the side toward (in) the hole, then there cannot be a sharp gradient. That this is the true situation is shown by a series of experiments with various concentrations of spores in synthetic medium in the test layers and non-spore synthetic medium in the opposite layers. Orientation improves with increasing spore concentration up to about 80 spores per mm.^{3} (Fig. 3). At greater spore concentrations the orientation of spores in the test region does not improve; higher levels of staling substance away from the hole are being matched by higher levels within the hole. (The same limitation applies to the method used in the studies of Graves (1916) and earlier workers, who did their experiments on mica plates with pin holes in them.)

If a plate is prepared with one of the "attractants" in the opposite layer, this material is in contact with the test layer within the hole, and during the incubation it diffuses out through the hole towards the test region. When it was found that these "attracting" materials showed pronounced effects only on plates with high spore concentrations in the test layer, it was suspected that they might function by inactivating staling substance in their presence. This would "flush" the staling substance within the hole, and permit a steep concentration gradient of this material to arise in the test region. The hypothesis is advanced, then, that these "attracting" materials act not by any true positive chemotropic effect on the spores, but only by inactivating a substance which does have a true tropic action.

As a further check of this hypothesis, test plates were set up with no spores in or above the hole. In this situation the test region becomes the very edge of the population (Fig. 1). This is accomplished by means of thumb tacks with the same bore as the holes in the plastic plates. The tacks are inserted in the holes before the test layer is poured, and after it has solidified they are removed, leaving a cylindrical hollow above each hole. When the plate is placed on the liquid, non-spore agar medium of the opposite layer, this material fills these hollows before it solidifies. When the growth on these preparations is examined, the test spores are found to show excellent orientation (15–20 degrees average), regardless of whether an "attractant" is present in the opposite layer or not. Thus the pronounced effect of the "attractants" seems to be directly dependent on the presence of spores within the hole.

A simpler test which indicates that this is not a true attraction is done with a layer of agar medium on a glass slide. Spores are inoculated only at one end, and the hyphae grow down the length of the slide. A block of the synthetic medium agar in the region ahead of the advancing hyphae is cut out and replaced by a block of turnip juice agar. If it were a true attractant, hyphae passing nearby should turn and grow into it, but they do not.

The experiments so far described fit very well the hypothesis that the "attracting" materials act only by inactivating staling substance in their presence. However, with undiluted tomato juice agar in the opposite layers of test plates, we observe marked improvement of orientation even when there is a low concentration of spores in the test layer. The method used in this experiment is not satisfactory in that it can never tell us whether the improved orientation results from an enhanced staling reaction or from a true positive tropic effect. This is because both of these possible effects would be working in the same direction in this system, and there is thus no visible way to distinguish between them. The same ambiguity exists, to a limited extent, in the preparations with no spores within the holes, though these were designed to minimize any effect on the staling reaction. The same is true for the experiment with a block of turnip juice agar in the neighborhood of growing hyphae.

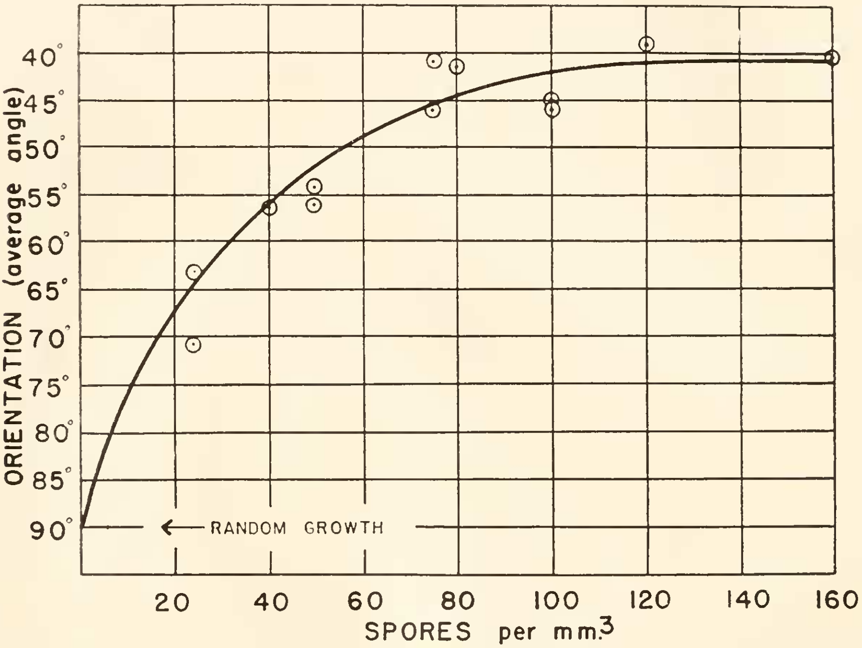

FIGURE 3. Orientation plotted against spore concentration for experiments with spores in synthetic medium in the test layer and non-spore synthetic medium in the opposite layer. Each point represents the average of four test regions.

A definitive test to learn the true nature of the effect can be designed by preparing the experiment in such a way that the "attractant" will be working in a direction antagonistic to the staling reaction. This is accomplished by putting the "attractant" material in the test layer with the spores, while the opposite layer contains non-spore synthetic medium. In this situation, the staling reaction tends to make the spores in the test region orient toward the hole, while the "attractant" tends to orient them away from the hole. If the experiment is prepared so that the staling reaction is very weak (low spore concentration), and the attraction is very strong (undiluted tomato juice), then we can tell whether it is a true attractant or a staling substance inactivator. If it is a true attraction, then it should completely outweigh the staling reaction in this situation and cause marked orientation away from the hole. If it works only by inactivating staling substance, then the most it can do is obliterate the staling reaction and cause random growth; 90 degrees becomes the limiting average angle. Table II shows the latter to be the case. With concentrated tomato juice on a series of plates with very low spore concentration, the average angle is below 90 degrees. With turnip juice, the result is the same. This is good evidence, then, that tomato juice and turnip juice, which showed the most marked effects of any of the materials in the previous tests, are not true chemotropic agents.

TABLE II

Orientation of spores in plant juice with synthetic medium in the opposite layer

"Attractant" in test layer	Spore concentration in test layer (per mm.^{3})	Number of test regions studied	Total no. of hyphae	Total angle	Average angle
Tomato juice	25	16	74	6000 degrees	81 degrees
Turnip juice	35	15	105	8950 degrees	85 degrees

Note: In the experiments reported in this table, all opposite layers contained non-spore synthetic medium.

Attempts at purification of the active material by extraction of yeast extract with a number of solvents (methanol, propanol, butanol and chloroform) have been unsuccessful. Paper chromatograms of yeast extract with a butanol-acetic acid mixture as the moving solvent affected some separation of components but did not result in any appreciable purification of the active material. Further chemical studies are projected with other solvents and perhaps other of the source materials.

The presence of a dialysis membrane between the two agar layers on the test plate does not hinder the effect of the "attractant" (from the Penicillium medium), but there is indirect evidence that the active material is slow-diffusing. The "attraction" phenomenon is qualitatively unchanged at any acidity permitting growth of the mold (pH 3 to pH 7).

DISCUSSION

The experiments which were done to determine the nature of the action of plant juices on orientation of the growth of Rhizopus spores demonstrated that the intensity of the plant juice effect varies with the amount of growing mold in its presence. The hypothesis has been advanced that the plant juices affect orientation by inactivating staling substance in their presence. It is also possible that they act by preventing the production of staling substance. A third possibility which fits the experimental findings equally well is that a true positive tropic agent is formed by the combination of a plant juice ingredient with some product of the mold's metabolism. Not knowing the chemical nature of the active plant juice material, it is difficult to design experiments which would discriminate between these various hypothetical mechanisms.

The present study does not enable us to evaluate the significance in nature of the tropic action of plant juices. Although these materials do not exercise direct attraction on the hyphae (of Rhizopus, at least), they do act in a way which could facilitate entrance into the stomata by germ tubes of spores germinating on the leaf surface. To understand the part played by chemical tropism in host-parasite relationships, a careful study should be made of the tropic sensitivities of a series of parasitic molds. If their tropic responses are similar to those of Rhizopus, then it does not seem possible that host specificity could be based on unique chemotropic sensitivities, as suggested by Massee (1905). In view of the many instances of specific growth factor requirements of molds which have been demonstrated in recent years, it appears more probable that this is the basis of host-parasite specificity.

Summary

Turnip juice and several other plant materials exert what appears to be a strong tropic attraction on the germinating spores of *Rhizopus nigricans* when tested on the type of experimental plates used in this and earlier studies. However, evidence is presented which demonstrates that the plant juices do not exercise a direct tropic action on the mold. It is suggested that these materials function by inactivating the staling substance (a negative tropic agent which is a normal product of the mold's metabolism).

LITERATURE CITED

Clark, J. F., 1902. On the toxic properties of some copper compounds with special reference to Bordeaux mixture. *Bot. Gaz.*, 33: 26–48.

Fulton, H. R., 1906. Chemotropism of fungi. *Bot. Gaz.*, **41**: 81–108.

Graves, A. H., 1916. Chemotropism in *Rhizopus nigricans*. *Bot. Gaz.*, **62**: 337–369.

Massee, G., 1905. On the origin of parasitism in fungi. *Phil. Trans. Royal Soc.* (*B*), **197**: 7–24.

Miyoshi, M., 1894. Ueber Chemotropismus der Pilze. *Bot. Zeitschr.*, **52**: 1–28.

Stadler, D. R., 1952. Chemotropism in *Rhizopus nigricans:* the staling reaction. *J. Cell. Comp. Physiol.*, **39**: 449–474.

Swingle, W. T., 1896. Bordeaux mixture. Bull. No. 9, Div. Veg. Phys. & Path., U. S. Dept. Agric.